THE APPLICATION OF ENZYMIC HYDROLYSIS AND TRITIUM LABELLING TO A STUDY OF THE MODIFICATION OF TRYPTOPHYL RESIDUES IN PROTEINS

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Abstract

The tryptophyl residues of apomyoglobin, *S*-carboxymethyl-lysozyme, and wool have been radioactively labelled in order to follow their reactions more easily. Whereas enzymic hydrolysis of these proteins gives good yields of tryptophan, it was found that enzymic hydrolysis of apomyoglobin, after either ozonolysis or treatment with *o*-nitrophenylsulphenyl chloride, released only part of the resultant modified tryptophyl residues. Enzymic hydrolysis gave a number of labelled products from the above proteins after irradiation with simulated sunlight, but only kynurenine could be identified. The isolation of yellow, radioactively labelled material from the enzymic hydrolysates of these irradiated proteins provides direct evidence that the yellow colour developed by irradiated wool and other proteins originates from tryptophyl residues.

I. INTRODUCTION

Procedures have been described recently for labelling the aromatic protons of the tryptophyl residues in proteins by treatment with tritiated trifluoroacetic acid (Bak *et al.* 1969; Holt *et al.* 1971). One of the main aims of our labelling experiments was to facilitate the isolation and identification of products arising from tryptophyl residues after irradiation of proteins. It was necessary to use enzymic hydrolysis, rather than acid hydrolysis, in this work because of the relative instability of tryptophan and many of its derivatives to acid hydrolysis. Although modified methods of acid hydrolysis minimize tryptophan degradation (Matsubara and Sasaki 1969; Liu and Chang 1971), they were still unsuitable for this study because of the instability of the incorporated tritium labels to strong acid (Holt and Milligan 1972). We have already developed an enzymic hydrolysis procedure which gives good yields of amino acids from the original tritiated proteins (Holt *et al.* 1971). However, it was necessary to determine the effectiveness of this procedure on modified proteins as a prerequisite to a study of irradiated proteins.

Apomyoglobin was chosen as a model and treated with two reagents known to specifically modify tryptophyl residues in proteins, viz. *o*-nitrophenylsulphenyl chloride (Boccu *et al.* 1970) and ozone (Previero *et al.* 1964; Morishita and Sakiyama

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1970). Having determined the limitations of our enzymic hydrolysis procedure in releasing the modified tryptophyl derivatives from these treated proteins, the same hydrolysis procedure was then applied to tritiated apomyoglobin, *S*-carboxymethyl-lysozyme, and wool after irradiation with simulated sunlight.

II. Materials and Methods

(*a*) *Materials*

Merino wool top (MW 148) was cleaned by successive extraction with light petroleum, ethanol, and water. Sperm whale myoglobin (Koch-Light) was freed from haem by the method of Teale (1959). *S*-Carboxymethyl-lysozyme was prepared from egg-white lysozyme (Sigma) by reduction and carboxymethylation according to O'Donnell and Thompson (1964).

Pronase AF (Kaken Chem. Co., Tokyo) was purified by fractional precipitation from aqueous acetone followed by chromatography on DEAE-cellulose. Leucine aminopeptidase and prolidase were commercial samples from P-L Biochemicals (Milwaukee, USA) and Miles Labs. (Kankankee, USA) respectively.

Tritiated trifluoroacetic acid was prepared by careful addition, with cooling, of an equimolar amount of tritiated water to freshly distilled trifluoroacetic anhydride. The specific activity of the resultant trifluoroacetic acid ranged from 1 to 45 mCi/mmole.

A sample of 2-(*o*-nitrophenylsulphenyl)-L-tryptophan was prepared according to Fontana *et al.* (1966).

(*b*) *Tritiation of Proteins*

Apomyoglobin and *S*-carboxymethyl-lysozyme (100 mg) were treated for 15 min at 20–25°C with an excess of tritiated trifluoroacetic acid (2–5 ml) containing 2-mercaptoethanol (0·02 ml), and then diluted with water (25–50 ml) and lyophilized. Dry wool (100 mg) was treated for 4 hr with tritiated trifluoroacetic acid (2 ml, without addition of 2-mercaptoethanol) and then washed repeatedly with water for 24 hr.

(*c*) *Treatment of Apomyoglobin with* o-*Nitrophenylsulphenyl Chloride* (*NPS-Cl*)

A stock solution of apomyoglobin (40 mg) in aqueous acetic acid (50% v/v, 4·0 ml) was used. Aliquots (1·0 ml) were treated at 20°C for 10 min with (1) NPS-Cl (0·3 mg) in acetic acid (0·5 ml), (2) NPS-Cl (150 mg) in acetic acid (0·5 ml), and (3) urea (0·4 g) followed by NPS-Cl (150 mg) in acetic acid (0·5 ml). At the completion of reaction the modified proteins were isolated by gel filtration on a column of Sephadex G25 resin equilibrated with 50% acetic acid, and made up to a volume of 10·0 ml with this solution. The optical densities of these solutions at 365 nm were then determined, using an aliquot (1·0 ml) of the original protein solution, diluted to 10·0 ml with 50% acetic acid, in the reference cell.

Subsequently, tritiated apomyoglobin was treated with NPS-Cl according to procedure (3) on five times the scale, and subjected to enzymic hydrolysis.

(*d*) *Ozonolysis*

A stream of ozonized oxygen was passed through a solution of tritiated apomyoglobin (50 mg) in anhydrous formic acid (25 ml) containing resorcinol (3 mg) for 10 min at 8°C (cf. Previero *et al.* 1964). The resultant solution was lyophilized and samples of the modified protein were then subjected to both acid and enzymic hydrolysis.

(*e*) *Analysis of Enzymic Hydrolysates*

Wool was reduced and carboxymethylated prior to hydrolysis. The procedures used for enzymic hydrolysis, for ion-exchange chromatography of the resultant hydrolysates, and for radioassay of the hydrolysis products have been described previously (Holt *et al.* 1971).

(f) Irradiations

The soluble proteins (35–40 mg) were dissolved in 0·02M HCl (80 ml) and irradiated in a 100-ml cell surrounding a jacketted 100-W Hanovia medium-pressure mercury lamp. Water at 20°C was circulated through the Pyrex jacket surrounding the lamp; only wavelengths > 295 nm were transmitted. Air was passed through the cell during irradiation (75 min).

The wool sample (150 mg) was spread over an area of 4 in.^{2}, wetted with distilled water, and placed 18 in. below an air-cooled Philips HOKI 2000-W mercury arc fitted with a Corning glass 7740 filter to exclude wavelengths below 300 nm, so as to simulate summer sunlight. The wool sample was maintained in a damp state while each side was irradiated for 24 hr.

III. Results and Discussion

As a preliminary to a study of irradiated proteins, apomyoglobin was treated with NPS-Cl to determine whether this modification of tryptophyl residues affects their subsequent release by enzymic hydrolysis. This combination of protein and reagent was chosen because NPS-Cl has been shown to modify tryptophyl residues in proteins specifically, provided no cysteine residues are present (Boccu *et al.* 1970). However, it was found that the tryptophyl residues of apomyoglobin were only partially modified under conditions where Boccu *et al.* observed complete modification for several other proteins. Fortunately, almost complete modification of the two tryptophyl residues in apomyoglobin could be achieved if the reaction with NPS-Cl was carried out in 4M urea. The extents of reaction after treatment under various conditions were determined by spectrophotometric analysis of the treated proteins at 365 nm and are shown in Table 1.

Table 1

Extent of reaction of *o*-nitrophenylsulphenyl chloride with the tryptophyl residues of apomyoglobin

Conditions*		NPS-Trp residues per molecule†
Moles NPS-Cl per Trp residue	Solvent	
3	50% CH_3COOH	0·8
150	50% CH_3COOH	1·15
150	50% CH_3COOH, 4M urea	1·9

* All treatments were carried out at 20–25°C for 10 min, and the protein was then isolated by gel filtration.

† Using a value of 4000 for the molar extinction coefficient of the 2-NPS-tryptophyl residue (cf. Boccu *et al.* 1970). There are two tryptophyl residues per molecule of apomyoglobin (Edmundson 1965).

(a) Enzymic Hydrolysis of Modified Apomyoglobin

The distribution of the radioactively labelled amino acids resolved by ion-exchange chromatography of enzymic hydrolysates of tritiated apomyoglobin, before and after treatment with NPS-Cl in the presence of urea, is shown in Figure 1. The major labelled amino acid obtained from the untreated protein was tryptophan,

accounting for 85% of the radioactive material eluted from the column. However, very little tryptophan was present in the enzymic hydrolysate of the NPS-Cl-treated apomyoglobin, most of the radioactive material being present in two yellow peaks, *A* and *B* (see Fig. 1). That in peak *B* behaved identically with an authentic sample

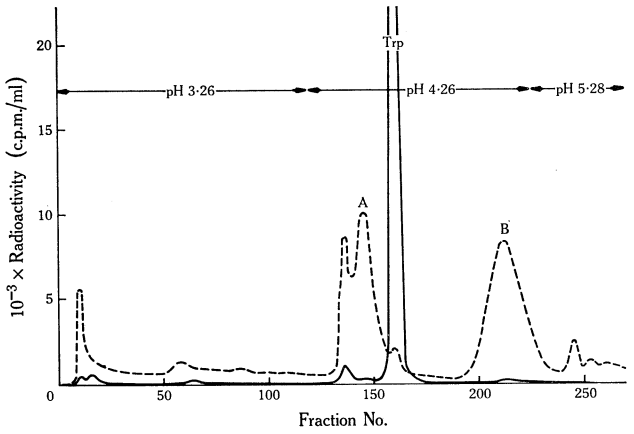

Fig. 1.—Ion-exchange chromatograms of enzymic hydrolysates of [^{3}H]apomyoglobin before (——) and after (- - - -) treatment with NPS-Cl in the presence of urea.

of 2-(*o*-nitrophenylsulphenyl)tryptophan (2-NPS-tryptophan) on ion-exchange chromatography and on paper electrophoresis at pH 1·9. Its specific activity was less than that of the original tryptophan, due to displacement of the tritium labels at the 2-position by the NPS-group. We have previously shown that 33–50% of the labels introduced into the tryptophyl residue in proteins by treatment with tritiated trifluoroacetic acid are present in the 2-position (Milligan *et al.* 1972). The material in peak *A* had an ultraviolet absorption spectrum very similar to that of 2-NPS-tryptophan, and is probably a mixture of peptides containing the 2-NPS-tryptophyl residue. The other radioactive peaks were not identified. Thus, NPS-tryptophan is more difficult to release from peptide combination than is tryptophan itself.

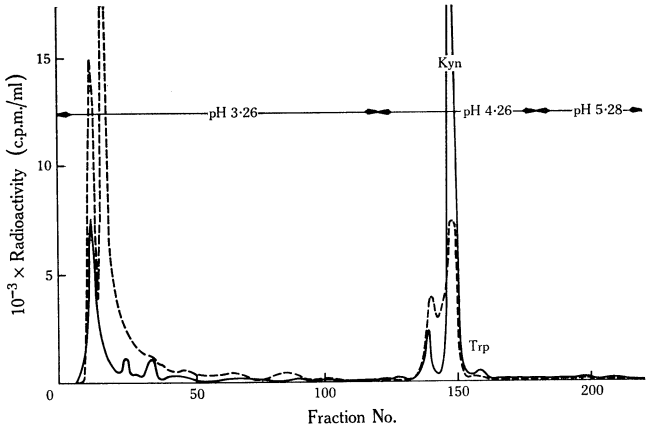

Fig. 2.—Ion-exchange chromatograms of acid (——) and enzymic (- - - -) hydrolysates of ozonized [^{3}H]apomyoglobin. The acid hydrolysis was carried out with 6M HCl at 105°C for 24 hr in an evacuated sealed tube.

Apomyoglobin was subjected to ozonolysis to determine whether the conversion of tryptophyl residues to *N*-formylkynurenine residues also adversely affected subsequent enzymic hydrolysis. Figure 2 shows that the major radioactive product

released by acid hydrolysis of ozonized tritiated apomyoglobin is kynurenine. Its identity was confirmed by comparison of its electrophoretic and chromatographic behaviour with that of an authentic sample. It accounted for 50% of the total radioactive material present. By comparison, enzymic hydrolysis released only a small amount of kynurenine (18% of the total radioactivity) and even less material running in the position of *N*-formylkynurenine (which is eluted at fraction No. 140).

Thus modification of tryptophyl residues in proteins renders their release by enzymic hydrolysis considerably more difficult than the release of the original tryptophyl residues. However, in the two cases studied the modified tryptophyl residues are released partially, permitting the use of the method for qualitative studies.

(*b*) *Enzymic Hydrolysis of Irradiated Proteins*

Irradiation of wet wool fabric and aerated aqueous solutions of apomyoglobin and *S*-carboxymethyl-lysozyme resulted in 49, 60, and 90% destruction of their tryptophyl residues, respectively. Ion-exchange chromatography of enzymic hydrolysates of the three irradiated proteins gave rise to a number of new peaks of radioactivity. These were best resolved in the case of *S*-carboxymethyl-lysozyme (see Fig. 3). A small peak, due to kynurenine, was present in each case but the

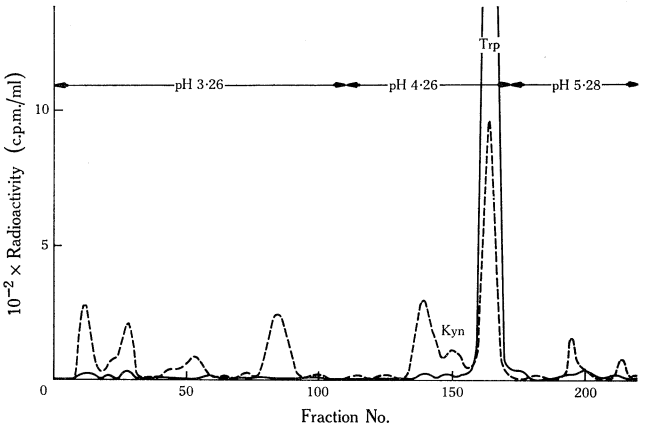

Fig. 3.—Ion-exchange chromatograms of enzymic hydrolysates of [^{3}H]*S*-carboxymethyl-lysozyme before (——) and after (- - - -) irradiation.

radioactive material present in the other peaks could not be identified. Kynurenine (or *N*-formylkynurenine) has previously been detected as an irradiation product of tryptophan (e.g. Asquith and Rivett 1971), tryptophyl peptides (e.g. Benassi *et al.* 1967), and tryptophan-containing proteins (Gomyo and Fujimaki 1970). Kynurenic acid, γ-(*o*-aminophenyl)homoserine, 3a-hydroxy-2,3,3a,8a-tetrahydropyrroloindole-2-carboxylic acid, and 4-(2′-amino-2′-carboxyethyl)quinazoline, which are potential photodecomposition products, were not present in detectable amounts in our enzymic hydrolysates.

With each protein hydrolysate the first peak of radioactivity to be eluted from the ion-exchange column was yellow, whereas all other fractions were colourless. Paper electrophoresis showed that each of these peaks contained a complex mixture of yellow radioactive compounds. Although attempts to fractionate larger amounts of this material by chromatography or gel filtration were unsuccessful, these experiments provide direct evidence that the yellow discoloration developed by wool during

irradiation arises from the tryptophyl residues. This substantiates earlier indirect evidence for this conclusion, based upon the correlation of rates of yellowing of wool and silk with rates of tryptophan destruction (Leaver and Ramsay 1969).

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